[14] Measurement of Cytosolic Free Ca^{2+} with Quin2

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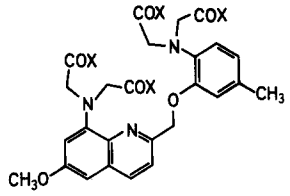
Introduction: General Advantages and Disadvantages

The fluorescent Ca^{2+} indicator dye, quin2 (Fig. 1), is the basis of one of the most popular methods for measuring cytosolic free Ca^{2+} [$(Ca^{2+}]_i$), especially in suspensions of mammalian cells. Briefer reviews of the use of quin2 have appeared previously.[1-3] Its widespread use probably stems from the following advantages that it has over other methods for measuring cytosolic free Ca^{2+}. (1) Quin2 can be loaded into the cytosol of millions of cells simultaneously, without any micromanipulations or any disruption of the plasma membrane.[4] Cells are simply incubated with quin2/AM, the tetra(acetoxymethyl) ester of quin2 (Fig. 1). This uncharged, hydrophobic derivative of quin2 diffuses freely across membranes but is gradually hydrolyzed by cytoplasmic esterases, eventually regenerating quin2 itself, which remains trapped in the cytosol as a relatively impermeant tetranion. All other methods, particularly those using ion-selective microelectrodes, aequorin, and arsenazo III, require breaching of the plasma membrane to enable the sensor molecules to contact the cytoplasm. Because of the enormous electrochemical gradient of Ca^{2+} across the plasma membrane, any threat to membrane integrity is bad for the cells and for the measurement. The nondisruptive method of loading quin2 via permeant hydrolyzable esters permits its use on cells too small or delicate to tolerate puncture or lysis and resealing. Also, the ability to measure an average $[Ca^{2+}]_i$ from large numbers of cells can be very helpful in establishing correlations of $[Ca^{2+}]_i$ with cell responses that must also be measured from whole populations, for example secretion, proliferation, or metabolism of cylic nucleotides, lipids, or proteins. (2) Quin2 is usually monitored with a conventional cuvette spectrofluorimeter. Both the dye and the instrumentation are widely available by purchase or loan from colleagues, whereas other methods tend to require much specialized or custom equipment. (3) Quin2 readings are usually easily calibrated at typical resting levels of $[Ca^{2+}]_i$ ($\sim 10^{-7}$ *M*) and below (to 10^{-8} *M*), whereas all other techniques do their best at detecting activated levels ($\sim 10^{-6}$ *M*)

[1] R. Y. Tsien, *Annu. Rev. Biophys. Bioeng.* **12,** 94 (1983).
[2] R. Y. Tsien, T. Pozzan, and T. J. Rink, *Trends Biochem. Sci.* **9,** 263 (1984).
[3] T. J. Rink and T. Pozzan, *Cell Calcium* **6,** 133 (1985).
[4] R. Y. Tsien, T. Pozzan, and T. J. Rink, *J. Cell. Biol.* **94,** 325 (1982).



quin2 : X= O^-
quin2/AM : X = OCH_2OCOCH_3

FIG. 1. Structure of Ca^{2+} indicator quin2 and its acetoxymethyl ester quin2/AM. Reproduced from Ref. 4, *The Journal of Cell Biology* (1982) 94:325–334, by copyright permission of the Rockefeller University Press.

and are nearly at their detection limits at resting levels. (4) Quin2 avoids many of the drawbacks particular to other $[Ca^{2+}]_i$ measurement techniques, for example, the irreversible destruction of aequorin by Ca^{2+}, the poor Mg^{2+} rejection and messy Ca^{2+} stoichiometry of arsenazo III and antipyrylazo III, the slow response time and need for voltage referencing of ion-selective electrodes, and the null point method's need to destroy large numbers of cells in low extracellular $[Ca^{2+}]$ for each data point.[1,5]

Of course, quin2 suffers from significant disadvantages, most of which were described in the first full articles[4,6] on the technique and which have been repeatedly rediscovered by subsequent authors. (1) The fluorescence of quin2 is not exceptionally bright, so that intracellular loading of several hundred micromolar is usually required for the quin2 signal to dominate cellular autofluorescence. This much quin2 can partially buffer $[Ca^{2+}]_i$ transients, though it generally does not alter $[Ca^{2+}]_i$ levels set by long-term homeostatic processes. This buffering can actually be extremely useful when examining whether $[Ca^{2+}]_i$ is really essential for a given cell response such as exocytosis or phosphoinositide breakdown.[6–9] $[Ca^{2+}]_i$ rises can often be suppressed to controllable extents by judicious use of varying quin2 loading, extracellular Ca^{2+} deprivation, and ionophore pretreatments. Comparisons of damped $[Ca^{2+}]_i$ signals and cell responses from the very same populations are a powerful tool to show whether $[Ca^{2+}]_i$ rises are really important, often revealing that they are

[5] R. Y. Tsien and T. J. Rink, *in* "Current Methods in Cellular Neurophysiology" (J. L. Barker and J. McKelvy, eds), pp. 249–312. Wiley, New York, 1983.
[6] T. Pozzan, P. Arslan, R. Y. Tsien, and T. J. Rink, *J. Cell Biol.* **94,** 335 (1982).
[7] F. DiVirgilio, D. P. Lew, and T. Pozzan, *Nature (London)* **310,** 691 (1984).
[8] T. J. Rink, S. W. Smith, and R. Y. Tsien, *FEBS Lett.* **148,** 21 (1982).
[9] L. M. Vicentini, A. Ambrosini, F. DiVirgilio, T. Pozzan, and J. Meldolesi, *J. Cell Biol.* **100,** 1330 (1985).

not as necessary as previously hypothesized. This seems an important and underused application of quin2, especially because other measurement techniques are much less amenable to variation in the extent of $[Ca^{2+}]_i$ buffering. But when rapid $[Ca^{2+}]_i$ transients are to be measured with minimum perturbation, an indicator working at lower loadings than quin2 would be highly desirable. (2) Obviously, quin2 is inappropriate for tissues that are highly opaque due to pigment or scattering or that are particularly sensitive to near-UV-light. (3) Because quin2 signals Ca^{2+} primarily by its fluorescence at a single set of excitation (339 nm) and emission wavelengths (490–500 nm), its signal becomes more difficult to calibrate in terms of absolute $[Ca^{2+}]_i$ values as one switches from suspensions to monolayers to single cells. A dye that shifted its preferred wavelengths rather than just changing its amplitude of fluorescence would be much easier to calibrate, since a mere change in dye content or instrumental sensitivity would not masquerade as a $[Ca^{2+}]_i$ change.[4,10] (4) Quin2 can be bleached by high illumination levels either in a cuvette or on a microscope.[11] (5) Quin2 does have some sensitivity to Mg^{2+}. Mg^{2+} by itself causes little change in the fluorescence with standard wavelengths of 339 nm excitation, but does act as a competitive inhibitor of Ca^{2+}-binding and associated fluorescence enchancement.[4,12] For a given error in estimating or guessing $[Mg^{2+}]_i$, quin2 at resting $[Ca^{2+}]_i$ levels would be much less affected than arsenazo III or an organophosphate-based ion-selective electrode, similarly or slightly less perturbed than aequorin would be, but more affected than an electrode using neutral carriers.[1,5] (6) A few tumor cell types have enough heavy metals in them to perturb the dye.[13] (7) Not all cells can be loaded by the gentle means of hydrolyzing membrane-permanent esters. Plant, bacterial, and a scattering of invertebrate cells load poorly or not at al,[14,15] whereas most vertebrate cells seem to load, with the possible exception of some but not all types of muscle. (8) In cells that do load by ester hydrolysis, the chemical by-products, formaldehyde and protons,[16] could have harmful side effects. (9) Quin2 is maximally sensitive to Ca^{2+} levels near its Ca^{2+} dissociation constant, $\sim 10^{-7}$ *M*;

[10] G. Grynkiewicz, M. Poenie, and R. Y. Tsien, *J. Biol. Chem.* **260,** 3440 (1985).
[11] B. A. Kruskal, C. H. Keith, and F. R. Maxfield, *J. Cell Biol.* **99,** 1167 (1984).
[12] R. Y. Tsien, *Biochemistry* **19,** 2396 (1980).
[13] P. Arslan, F. DiVirgilio, M. Beltrame, R. Y. Tsien, and T. Pozzan, *J. Biol. Chem.* **260,** 2719 (1985).
[14] R. J. Cork, *Plant Cell Environ.* **9,** 157 (1986).
[15] J. I. Korenbrot, D. L. Ochs, J. A. Williams, D. L. Miller, and J. E. Brown, *in* "Optical Methods in Cell Physiology" (P. DeWeer and B. M. Salzberg, eds), pp. 347–363. Wiley, New York, 1986.
[16] R. Y. Tsien, *Nature (London)* **290,** 527 (1981).

much higher levels ($>10^{-6}$ *M*) nearly completely saturate the dye and cannot be distinguished from each other.[4] Moreover, if the dye resides in several compartments with different $[Ca^{2+}]_i$, a measurement that ignores the compartmentation will underestimate the deviations from the mean.[6]

Many of the above problems can be alleviated by more recently introduced relatives of quin2. These newer fluorescent indicators,[10] especially fura-2 and indo-1, are much brighter in fluorescence than quin2. Because so much less dye needs to be introduced, both the Ca^{2+} buffering and the potential for toxicity from hydrolysis products are greatly reduced. The new dyes shift wavelengths not just intensity upon binding Ca^{2+}, so calibration of signals from monolayers and single cells is greatly eased. Fura-2 is much more resistant to photobleaching[17] than quin2 and has better selectivity for Ca^{2+} over Mg^{2+} and heavy metals. Because fura-2 and indo-1 are sensitive enough to give large calibratable signals from single cells or regions of cells,[18–20] they allow direct measurement of compartmentalization and heterogeneity. For those reasons, we expect quin2 eventually to be replaced in most applications by fura-2, indo-1, or even newer relatives under development. This article, written in 1985–1986, concentrates mainly on quin2, the longer established indicator, whereas the newer dyes are discussed in more recent reviews.[21–25] Many of the protocols and cautions described below are similar for all the dyes. Moreover, to take full advantage of the advanced properties of the new dyes does require more elaborate equipment, so many newcomers to the field of $[Ca^{2+}]_i$ measurement will still want to get started using quin2 in a conventional cuvette fluorometer.

Dye Loading

The protocol for loading quin2 into intact cells was originally described in lymphocytes[4,26] and has been successfully applied with minor modifications to a variety of cell types, mostly mammalian. Basically,

[17] D. A. Williams, K. E. Fogarty, R. Y. Tsien, and F. S. Fay, *J. Gen. Physiol.* **86,** 37a (1985).
[18] R. Y. Tsien, T. J. Rink, and M. Poenie, *Cell Calcium* **6,** 145 (1985).
[19] D. A. Williams, K. E. Fogarty, R. Y. Tsien, and F. S. Fay, *Nature* (*London*) **318,** 558 (1985).
[20] M. Poenie, J. Alderton, R. Steinhardt, and R. Y. Tsien. *Science* **233,** 886 (1986).
[21] R. Y. Tsien and M. Poenie, *Trends Biochem. Sci.* **11,** 450 (1986).
[22] P. H. Cobbold and T. J. Rink, *Biochem. J.* **248,** 313 (1987).
[23] R. Y. Tsien, *Trends Neurosci.* **11,** 419–424.
[24] R. Y. Tsien, *Methods Cell Biol.* **30,** 127 (1989).
[25] R. Y. Tsien, *Annu. Rev. Neurosci.* **12,** in press.
[26] R. Y. Tsien, T. Pozzan, and T. J. Rink, *Nature* (*London*) **295,** 68 (1982).

quin2/AM from a stock solution in dimethyl sulfoxide (DMSO) is added to cell suspensions or monolayers. After some time the cells are washed, resuspended in fresh medium, and recording of fluorescence is initiated. Major variables in this protocol are (1) source of quin2/AM, (2) quin2/AM concentration and cell density, (3) incubation time and temperature, and (4) addition of serum or albumin.

Source

Quin2 and quin2/AM are now available from several commercial sources: Lancaster Synthesis, Amersham, Calbiochem-Behring, Sigma, Aldrich, and Dojindo Laboratories. The first five firms have branches both in the United States and Europe, while Dojindo is at 2861 Kengun-machi, Kumamotoshi (862), Japan. [It is our understanding that Amersham nonradioactive material and Sigma are merely repackagings of the Dojindo material.] We have not seen any consistent difference in quality between products from different companies, though there have been several isolated incidents of bad batches. Quin2 free acid is a free-flowing powder; its color is white or yellow, depending on purity and degree of protonation. Quin2/AM is a yellow gum or resin that should be fairly hard at refrigerator temperature but usually becomes sticky at room temperature. Routinely we keep both compounds desiccated over silica gel at −20° for long-term storage, since the protonated free acid can gradually decarboxylate and the AM ester can hydrolyze and/or oxidize. (The only derivative known to be crystallizable and indefinitely stable at room temperature is the tetraethyl ester, which is still the best source for critical studies on quin2 tetranion.)

Tests for the quality of quin2, in order of increasing laboriousness and stringency, are determination of extinction coefficient ($\varepsilon = 5000\ M^{-1}\ cm^{-1}$ at 354 nm in Ca^{2+}- and Mg^{2+}-free medium above pH 7.0 with millimolar EDTA or EGTA to chelate contaminating Ca^{2+}), determination of fluorescence excitation spectra before and after addition of excess Ca^{2+}, and determination of K_D for Ca^{2+}. Measurement of the extinction coefficient tests for gross inert contamination. Fluorescence excitation spectra at micromolar dye concentrations in high Ca^{2+} should show a peak near 339 nm excitation, whose amplitude above blank background is five- to seven-fold greater than the amplitude above background of the same dye concentration at the same wavelength in zero Ca^{2+} with EDTA or EGTA. Examples of such excitation spectra at saturating and zero Ca^{2+} are shown in Fig. 2 as the two extremes of the series of spectra. If adequate signal amplitude or stability cannot be attained, either the dye or fluorometer could be deficient; if an adequate enhancement due to Ca^{2+} cannot be

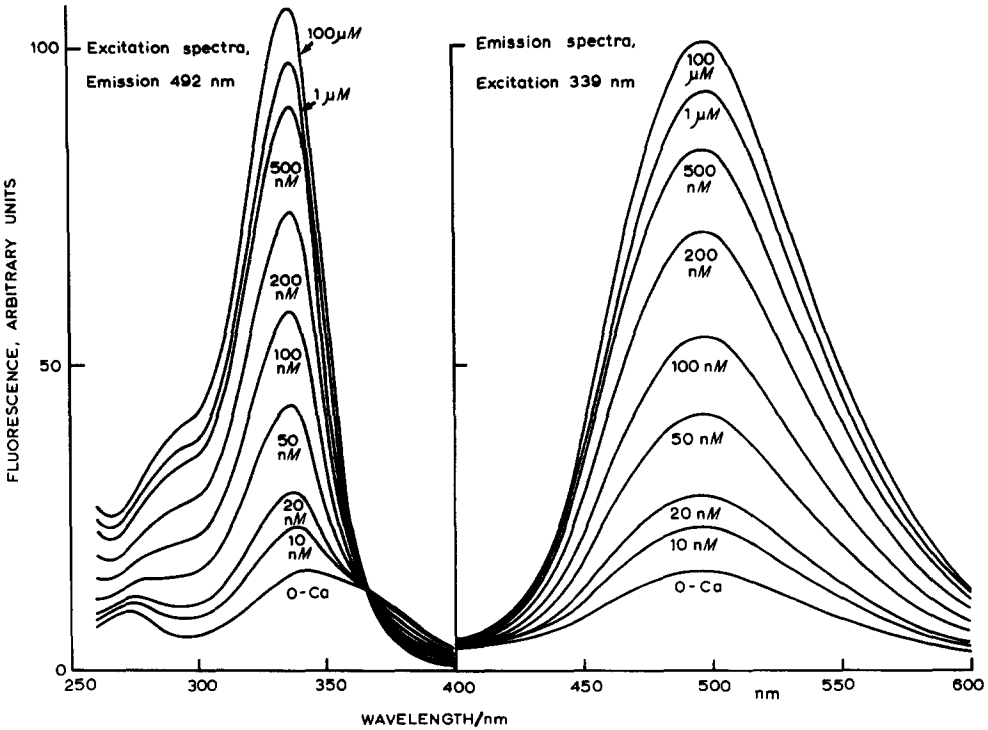


FIG. 2. Excitation and emission spectra of 20 μM quin2, with varying $[Ca^{2+}]$ as shown against an ionic background of 120–135 mM K^+, 20 mM Na^+, 1 mM free Mg^{2+}, and pH 7.05 at 37°. Reproduced from Ref. 4, *The Journal of Cell Biology* (1982) 94:325–334, by copyright permission of the Rockefeller University Press. Further experimental details may be found in that article.

found with a small excitation bandpass (<5 nm), then bad dye should be suspected. Measurement of the K_d for Ca^{2+} using intermediate buffered Ca^{2+} values (as in Fig. 2 and the section Calibration Procedure: Quin2 Affinity for Ca^{2+}, below) tests that all the molecules have the same high-affinity binding site; if some molecules lack one or more carboxylates, they would have lower affinities, yet would show the same spectra at zero and very high Ca^{2+}.

Unlike quin2, the ester quin2/AM is much more soluble in organic solvents than in water and has no Ca^{2+}-binding properties. When one has access to a cell system known to hydrolyze quin2/AM, the latter is usually tested merely by its ability to load quin2 into the cells. If other tests are required, thin-layer chromatography on silica gel is convenient. A solution of the dye in chloroform or dichloromethane is spotted on a

Merck 5554 silica gel TLC plate, 10 cm long, developed with hexane–ethyl acetate (1 : 1, v/v), and visualized under 254 or 365 nm illumination. Quin2/AM runs with R_f = 0.27 in this system. Unfortunately DMSO perturbs this R_f. Minor fluorescent contaminants are often present and usually tolerable. Yet another test of quin2/AM is to hydrolyze the dye chemically. Thus a small aliquot of a 1–10 m*M*, quin2/AM solution in DMSO is mixed with an equal volume of 0.1–1 M aqueous NaOH or KOH. After waiting 5–10 min, the mixture should be a stock solution of quin2 tetranion which can be tested as described above. Of course, base hydrolysis does not test the completeness or correctness of esterification.

Quin2/AM stock solutions of 1–10 m*M* concentration are usually made in DMSO because of its ready miscibility with water, biological inertness in most systems, nonvolatility, and ease of microliter pipetting. The DMSO should be anhydrous, and stock solutions of quin2/AM should be kept frozen and desiccated. Because the melting point of DMSO (18°) is sharply depressed by water content, moisture pickup is easily seen as rapid melting of the solution upon removal from the refrigerator.

Quin2/AM Concentration and Cell Density

Typically, the 1–10 m*M* stock solution of quin2/AM in DMSO is diluted 100- to 1000-fold into the cell suspension while stirring or shaking. Depending on cell type and other variables discussed below, typically 5–40% of the total quin2/AM added will become trapped as quin2 inside the cells. Since the cells usually occupy 0.1–1% of the volume of the suspension, intracellular quin2 concentrations reach 0.1 to 5 m*M*, substantially higher than the 1–100 μM initial concentration of quin2/AM in the suspension.

Quin2/AM before hydrolysis is actually quite hydrophobic and poorly soluble in simple salines. Light-scattering measurements (T. Pozzan, unpublished observations) suggest that above a few micromolar, the ester forms colloidal suspensions or precipitates. Therefore loading efficiency, or the percentage of quin2/AM usefully trapped, is improved by squirting the dye solution directly into a dense and stirred suspension of cells.[4] High concentrations of dye ester without sufficient cells or protective proteins simply precipitate dye wastefully, some of which may then be endocytosed and generate undesired intracellular background fluorescence.

Though loading efficiency is improved by high initial cytocrits, nearer 1% than 0.1%, the cells soon acidify the medium. Also, the unavoidable death of a fraction of the cells may release enzymes that can harm other

cells and hydrolyze extracellular quin2/AM. Therefore, dense suspensions should be diluted up to 10-fold a few minutes after the ester is added, since that interval seems sufficient to complete the initial association of the ester with cells, though not the full hydrolysis.

Incubation Time and Temperature

Quin2/AM is transformed to quin2 tetranion by four separate hydrolysis steps, one for each acetoxymethyl group. It is essential that this process be completed before measurements are begun. Any dye molecule with incompletely hydrolyzed ester groups contributes fluorescence yet is crippled in its Ca^{2+}-binding capability, leading to an underestimate of $[Ca^{2+}]_i$. If hydrolysis continues during the observation of fluorescence, the gradual increase in Ca^{2+} affinity will cause a rising curve that will simulate a progressive rise in $[Ca^{2+}]_i$. To aid completion of hydrolysis, it is important that the free concentration of the starting tetraester be reduced practically to zero well before observation begins. Otherwise the cells never get a chance to clear the metabolic pipeline of partially hydrolyzed intermediates. In dense suspensions of cells with high esterase activity, as in our initial studies with blood-forming cells, this exhaustion of quin2/AM is automatic as the cells take up and hydrolyze the ester. In some cases precipitation can help reduce the quin2/AM concentration. But if cell density or esterase activity is low, the experimenter should help by washing the cells with ester-free media after the initial contact period of a few minutes to 30 min. The cells should then be incubated in dye-free media until complete hydrolysis of the trapped intermediates which still bear one, two, or three ester groups. A quick and crude indication[4] of hydrolysis is a shift in the emission peak from 430 to 490–500 nm, matching that of the dye tetranion with or without Ca^{2+}. However, this widely used test is not fully rigorous, because an acetoxymethyl group lingering on the benzene ring portion of quin2 would not affect the fluorescence properties of the quinoline portion. The most rigorous test for full hydrolysis is to release the dye by lysing the cells, then titrate the dye with known buffered Ca^{2+} levels and quantitatively verify its responsiveness.[4] Obviously, such titration does not have to be done routinely, but should be checked during the establishment of a loading protocol for the particular tissue being studied.

We and most others have allowed about 1 hr incubation for full hydrolysis of the four ester groups. Some cells apparently hydrolyze the ester faster (for example, hepatocytes load well in just 2.5 min[27]), some more

[27] A. Binet, B. Berthon, and M. Claret, *Biochem. J.* **228,** 565 (1985).

slowly (i.e., some cultured cell lines), some not at all. In most cases the loading temperature has been 37°. There are a few reports, mainly in monolayers, where the loading was performed at room temperature. One of us (TP) has observed in brain synaptosomes, neutrophils, and the insulinoma cell line RINm5F, that loading at temperatures below 37° seems to result in trapping of quin2/AM in cell compartments different from the cytoplasm. In these cases, the emission spectrum retains a significant shoulder at 430 nm, indicating noncomplete hydrolysis. This shoulder surprisingly cannot be abolished either by prolonging the incubation time or by rewarming the cell to 37°. Another sign of such compartmentation is that digitonin treatment, which normally releases most cellular quin2, releases a smaller fraction (70–80%) of the dye; the residue requires Triton X-100 for release.

Albumin and Serum during Loading

Inclusion of either bovine serum albumin (0.5–1%) or fetal calf serum (1–5%) in the loading medium generally tends to increase the loading efficiency, probably because the proteins reduce the precipitation of quin2/AM and act as buffers for it. Moreover they increase the viability of most cell types. Very high concentrations of protein, as in platelets in their own plasma, may somewhat reduce loading efficiency by excessive competition for the dye, but the minimization of cell manipulation more than outweighs the waste of dye (T. J. Rink, personal communication). BSA and FCS can have some fluorescence of their own, and definitely reduce the potency of calcium ionophores,[28] so they are omitted or minimized in the final medium during actual measurements.

Recording the Fluorescence

Once dye loading is complete, the cells are typically washed once or twice to remove leaked or excess dye and hydrolysis by-products. Resuspension for fluorescence measurements should be in a medium containing as little as possible of such absorbing and fluorescing substances as phenol red, fetal calf serum, tryptophan, riboflavin, and pyridoxine. Cells that are not to be used immediately are resuspended in whatever medium at whatever temperature will best preserve their viability and dye content; often, room temperature is suitable. If the cells have been kept for a considerable time, a final wash just before use is helpful again to remove leaked dye and fluorescent additives from the medium.

[28] L. O. Simonsen, *J. Physiol. (London)* **318**, 34P (1981).

A wide variety of commercially available spectrofluorometers have been used successfully with quin2 in cell suspensions in cuvettes, so instrumental requirements are not stringent. However, it is important to scan the excitation and emission spectra to verify the quality and rate of dye loading. Therefore, filter fluorometers working at fixed wavelengths are not adequate unless the user is very experienced with quin2 and the tissue particularly tractable. Probably the most important characteristics of a good spectrofluorometer for quin2 studies are stability of output signal over the time scale of an experiment, and ability of the sample compartment to maintain whatever thermostatting, gassing, and/or stirring are needed for the particular cell type. Instrumental stability is easily tested by placing an unstirred solution of quin2 free acid in buffer of known high or low $[Ca^{2+}]$ and looking for an unchanging signal amplitude. Assuming temperature equilibrium has been reached, any progressive decline in signal suggests a failing instrument, or more likely, dye bleaching due to excessive illumination intensity. Bleaching is confirmed if the signal stops declining or even partially recovers either upon stirring the solution or temporarily blocking the illumination. Bleaching can be minimized by reducing the monochromator slit settings or inserting filters, screens, or apertures in the excitation beam. Most commercial instruments have enough sensitivity if in good repair to reach a stable and sufficiently quiet signal with excitation intensity low enough to avoid significant bleaching over the 0.5–1 hr time scale. Very high sensitivity is not required, since intrinsic autofluorescence from the cells is usually readily detectable at moderate gain and one generally has to load enough dye (usually several hundred micromolar) to overcome autofluorescence by a factor of two or more.

Thermostatting and gassing of the cuvette are obvious requirements for many cell preparations. Continual stirring is often unnecessary with small and well-dispersed cells, which settle sufficiently slowly so that occasional manual stirring with a Pasteur pipette is adequate. However, larger cell or clumps of cells need continual stirring. Magnetic drive is better than an overhead paddle because addition of solutions is unobstructed.

Traditional 1 cm square quartz cuvettes are expensive, fragile, and very wasteful of solution volume and cells. We find that cheap round shell vials or test tubes made of borosilicate glass are usually adequate[3] except for the most precise physicochemical measurements. The glass walls are thin enough to absorb a negligible amount (a few percent) of the incident UV beam. The cylindrical shape reduces wasted volume in the corners, improves stirring, yet seems not to cause optical problems. Depending on precise dimensions, sample volumes one-fourth to one-half that of 1 cm^2

cuvettes are sufficient, reducing usage of tissue and dyes correspondingly. The vials are rugged and cheap enough to be centrifugable, a help when assessing leakage. The main disadvantage of the vials is the need to build a metal adapter to center the vial in a conventional square sample holder.

Quin2 has also been used in cell monolayers grown on glass coverslips.[29] The coverslip is clamped by spring tension in a holder that fits in a square cuvette and maintains the coverslip at a near-diagonal orientation to the fluorimeter beams. Coverslips obviously are advantageous for anchorage-dependent cells; also they permit complete *in situ* replacement of the solution, whereas one can add to but not subtract from a cuvette of suspended cells. However, stability of the fluorescence amplitude is more difficult with coverslips, since minute motions of the coverslips, bleaching in the fixed zone of illumination, and detachment of cells can cause severe artifacts. Some success has been reported[30] in growing anchorage-dependent cells on microcarrier beads and suspending them in cuvettes, but this variant has not achieved popularity, perhaps because the considerable light scattering from the beads tends to contaminate or obscure the fluorescence signal, and the vigorous stirring needed to keep the beads in suspension tends to scrape the cells off.

Calibration Procedure

The fluorescence signal recorded from loaded cells reflects not only some sort of mean cytosolic free Ca^{2+} concentrations, $[Ca^{2+}]_i$, but also many other factors. These necessarily include the effective affinity of quin2 for Ca^{2+}, the concentration of quin2 within the cells, the number of cells in the effective sample volume, and the sensitivity of the fluorometer. Furthermore, if any significant quantity of dye is present in noncytoplasmic compartments such as extracellular space or intracellular organelles, such dye will contaminate the overall fluorescence signal. The purpose of calibration is to give a quantitative estimate of $[Ca^{2+}]_i$ that is as independent of the other variables as possible. We first discuss the determination of the effective affinity of quin2 for Ca^{2+}, then the several methods available for handling uncertainties in dye content, location, and instrumental sensitivity. Operationally, calibration is a much simpler procedure than one might think from the length of the following discussion, which attempts to summarize the major alternative procedures and their implicit assumptions.

[29] W. H. Moolenaar, L. G. J. Tertoolen, and S. W. deLaat, *J. Biol. Chem.* **259,** 8066 (1984).
[30] J. D. R. Morris, J. C. Metcalfe, G. A. Smith, T. R. Hesketh, and M. V. Taylor, *FEBS Lett.* **169,** 189 (1984).

Quin2 Affinity for Ca^{2+}

Ideally, one would determine the dissociation constant (K_D) of the quin2-Ca^{2+} complex in actual cytoplasm. However, this would require a highly accurate method for setting or reading $[Ca^{2+}]_i$ independently of quin2 itself. Such methods are rarely available in the cell systems to which quin2 is most suited. Instead one has to determine the K_D *in vitro* in buffers believed to mimic the main ionic constituents of cytoplasm, as described below. Are these *in vitro* K_D values applicable to cytoplasm? Probably yes, because quin2 estimates of $[Ca^{2+}]_i$ are rather close to those obtained by quite independent techniques. For example, estimates of resting $[Ca^{2+}]$ in toad stomach smooth muscle[19] cells are 129 ± 4 n*M*, 137 ± 13 n*M*, and 148 ± 20 n*M* by quin 2, fura-2, and ion-sensitive microelectrodes, respectively. In hepatocytes, quin2 values of 200 n*M*[31] and 160 n*M*[27,32] compare well with 211 ± 9 n*M* and 190 ± 10 n*M* using aequorin[33] and null points,[34] respectively.

The *in vitro* affinity of quin 2 for Ca^{2+} is determined by setting free Ca^{2+} to a series of accurately known values, measuring the dye fluorescence at each value, and fitting the data to the theoretical equation for one-to-one binding.

$$F = F_{min} + (F_{max} - F_{min}) \left(\frac{[Ca^{2+}]}{[Ca^{2+}] + K_D} \right)$$

Examples of the fluorescence data and the fit are shown in Figs. 2 and 3. This titration was done against a constant background of 120 m*M* K^+, 20 m*M* Na^+, 1 m*M* free Mg^{2+}, pH 7.05, at 37°, an ionic milieu believed to correspond in major cation composition to lymphocyte cytoplasm.[4,35] Nearly all other investigators using quin2 have continued to use the K_D, 115 n*M*, obtained from Fig. 3. However, the effective K_D is definitely sensitive to ionic strength, temperature, free Mg^{2+}, and pH values if they fall below about 6.5–6.8. In addition, any deterioration of the dye sample or incompleteness of ester hydrolysis will generate chemical heterogeneity that can jeopardize the quantitative calibration. Therefore it is highly desirable, though admittedly tedious, to generate the equivalent of Figs. 2 and 3 on lysates of loaded cells from each new tissue system being studied, with the correct temperatures and ionic background for that system.

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[32] A. P. Thomas, J. Alexander, and J. R. Williamson, *J. Biol. Chem.* **259,** 5574 (1984).

[33] N. M. Woods, K. S. R. Cuthbertson, and P. H. Cobbold, *Nature (London)* **319,** 600 (1986).

[34] E. Murphy, K. Coll, T. L. Rich, and J. R. Williamson, *J. Biol. Chem.* **255,** 6600 (1980).

[35] T. J. Rink, R. Y. Tsien, and T. Pozzan, *J. Cell Biol.* **95,** 189 (1982).

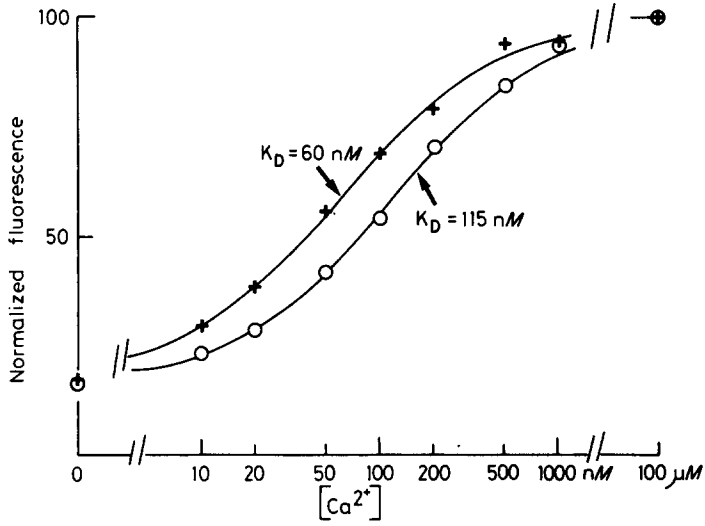

FIG. 3. Quin2 calibration curves in 120–135 m*M* K^+, 20 m*M* Na^+, pH 7.05, at 37°, and two different $[Mg^{2+}]$ values. Normalized quin2 fluorescence (excitation 339 nm; emission 492 nm) plotted against free $[Ca^{2+}]$ at 0 Mg (crosses) and 1 m*M* free Mg^{2+} (circles). The data points are fitted with curves corresponding to one-to-one stoichiometry and the indicated apparent dissociation constants. Reproduced from Ref. 4, *The Journal of Cell Biology* (1982) 94:325–334, by copyright permission of the Rockefeller University Press.

Ionic Strength and Monovalent Cation Concentration. So far, quin2 has been mostly used in mammalian cells, though a few reports of its application to other eukaryotic cells have also been published. In mammalian cells it is likely that the ionic strength and total monovalent cation concentration are normally rather constant. However, a change in ionic strength from that typical of a mammalian cell to that typical of marine invertebrate axoplasm increases the Ca^{2+} dissociation constant of related tetracarboxylate chelators by three- to fourfold.[10] Though we have not measured the quin2 K_D at high ionic strength, we would expect the same increase to apply.

Temperature. Increasing the temperature moderately increases the dissociation constant for Ca^{2+}, but a more significant effect is to decrease the fluorescence intensity of quin2 at fixed $[Ca^{2+}]$. A change from 20° to 37° decreases the fluorescence intensity of quin2 by a factor of approximately 2. This effect explains the common experience that cold solutions that are put in a warm thermostatted cuvette often decrease in fluorescence for the first few minutes before reaching a steady baseline level.

pH. Quin2 and other tetracarboxylate Ca^{2+} indicators are much less sensitive to pH changes around 7 than their parent EGTA is. This is because the highest pK_a of quin2 is ≤ 6.5 compared to 9.58 for EGTA. The effective K_D of quin2 for Ca^{2+} is increased by acidification of the medium below pH 6.8, while above this value pH changes are practically ineffective. In lymphocytes we measured[35] a resting pH_i of 7.05 for $pH_o = 7.4$, hence the choice of pH for Fig. 2. Since in most mammalian cells pH_i has been shown to range between 6.9 and 7.4, pH_i is probably not a major source of error in the calibration of $[Ca^{2+}]_i$. There are, however, some manipulations or drugs which can acidify pH_i considerably, which would complicate their effects on quin2 signals. Examples are mitochondrial uncouplers, anoxia, and permeant weak acids.

$[Mg^{2+}]_i$. There are many fewer direct measurements of free $[Mg^{2+}]_i$ than of pH_i. Our measurement of about 1 m*M* in lymphocytes[35] was the basis for the choice of $[Mg^{2+}]$ in Fig. 2. The affinity of quin2 for Mg^{2+} is in the 1–2 m*M* range, so an increase of Mg^{2+} can significantly increase the effective K_D of quin 2 for Ca^{2+}. Thus a change of Mg^{2+} from 0 to 1 m*M* results in a increase of the apparent K_D of quin 2 for Ca^{2+} from 60 to 115 m*M*, respectively.[4] Fortunately, the standard excitation wavelength of 339 nm not only maximizes the effect of Ca^{2+} on the fluorescence but also minimizes the direct effect of Mg^{2+} on the fluorescence, which is why 339 mn was chosen. At other wavelengths Mg^{2+} directly affects the spectrum as well as competing for Ca^{2+} (Fig. 4). There is little evidence up to now for changes of $[Mg^{2+}]_i$ upon cell stimulation, but the possibility of variability of the $[Mg^{2+}]_i$ between cell types has to be remembered.

Quin2 Localization in Cells

One of the most attractive features of the methodology is that most if not all of the trapped quin2 seems to reside in the cytoplasm or nucleus as opposed to other organelles. This generalization seems valid for the cells we have investigated, i.e., lymphocytes, platelets, neutrophils, Ehrlich and Yoshida carcinomas, and the cell lines PC12, RINm5F, and HL60. However, few workers have investigated in detail the localization of quin2 within other cell types, which may act differently.

Intracellular localization has been determined in various ways: (1) Direct morphological observation by fluorescence microscopy[11] reveals mainly diffuse rather than punctuate fluorescence, suggesting a cytoplasmic localization. (2) Detergents like digitonin, which preferentially permeabilize membranes with high-cholesterol content (like the plasma membrane), release practically all trapped quin2 in parallel to cytoplasmic

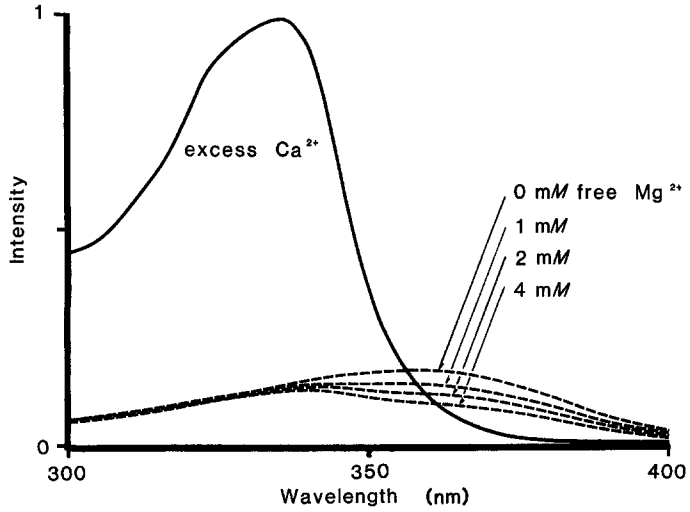

FIG. 4. Effect of Mg^{2+} on the excitation spectrum of quin2. Though Mg^{2+} has little effect at 335–340 nm, it significantly depresses the excitation amplitude at 350–370 nm, so that the ratio of the excitations at these two bands can be perturbed by physiological $[Mg^{2+}]$. The solution contained 10 μM quin2 in 132 mM KCl, 1 mM K_2H_2EGTA, 10 mM K-MOPS, pH 7.18, at 20°. $MgCl_2$ was added to reach 1, 2, and 4 mM free $[Mg^{2+}]$, then 4 mM $CaCl_2$ was added to saturate the dye with Ca^{2+}. Excitation bandpass was 1.8 nm; emission was collected at 500 nm with 9.25 nm bandpass. Excitation spectra were corrected by a rhodamine B quantum counter.

markers, with minor release of enzymes from mitochondria, lysosomes, and endoplasmic reticulum.[4,13,36,37] (3) High voltage discharge, which makes small holes in the plasma membrane, releases most intracellular quin2.[4] (4) Incubation of quin2/AM with partially purified cellular fractions reveals that the relevant esterase activity is localized in the soluble cytosolic fraction.[4] (5) Measurement of extracellular quin2 after strong stimulation of secretion indicates that, in platelets[8] and neutrophils,[36] quin2 is not appreciably contained in secretory granules. However, it appears that mast cell granules can pick up substantial amounts of fluorescence.[38]

In lymphocytes we also found[4] that the nuclear membrane does not constitute a permeability barrier for quin2, so that the dye is neither concentrated nor excluded from the nucleus. Since most evidence had suggested that ionic activities are the same in the nucleus as in the cyto-

[36] T. Pozzan, D. P. Lew, C. Wollheim, and R. Y. Tsien, *Science* **221,** 1413 (1983).
[37] J. Meldolesi, W. B. Huttner, R. Y. Tsien, and T. Pozzan, *Proc. Natl. Acad. Sci. U.S.A.* **81,** 620 (1984).
[38] W. Almers and E. Neher, *FEBS Lett.* **192,** 13 (1985).

plasm, we felt that the nucleus could be considered as part of the cytoplasm as far as quin2 and Ca^{2+} were concerned. However, recent evidence suggests that, in toad stomach smooth muscle cells, the nucleus preferentially accumulates dye[39] and may regulate Ca^{2+} to a level different from the cytoplasm.[19] Obviously this question needs reexamination in other cell types.

Calibration by Lysis

This procedure has been adapted from the calibration commonly used[40] for the Ca^{2+}-sensitive photoprotein, aequorin. The cells are lysed at the end of the experiment, usually with a detergent, to release all the indicator into the medium where Ca^{2+} is known to be high (for example, 1 m*M*). The fluorescence F_{max} thus obtained represents the fluorescence intensity of the dye at saturating Ca^{2+}. Ca^{2+} in the medium is then chelated with excess EGTA and base to establish F_{min}, the fluorescence of the dye at zero Ca^{2+}. Any fluorescence value F intermediate between F_{min} and F_{max} corresponds to a free $[Ca^{2+}]$ given by the following calibration equation:

$$[Ca^{2+}]_i = K_D \left(\frac{F - F_{min}}{F_{max} - F} \right) \quad (1)$$

Assumptions in this equation are that all the dye molecules share the same dissociation constant K_D for binding Ca^{2+} in a 1 : 1 complex, that the fluorescence is a linear function of the concentrations of Ca^{2+} bound and unbound dye, that any cellular autofluorescence has either been deducted or is unaffected by lysis, and that the instrument's sensitivity to dye fluorescence is not affected by lysis. This last assumption should be true in a dilute well-stirred cell suspension in a cuvette, since the average number of dye molecules in the macroscopic light path of the spectrofluorometer is the same before or after lysis. However, this assumption is insecure when a cell monolayer is held at the center of the cuvette, since lysis allows dye to spread into nonilluminated corners of the cuvette. The assumption is even worse when single cells are viewed by microscopy.

As discussed below in the section Correction for Extracellular Dye, Autofluorescence, and Heavy Metals, it is often advantageous to lower external Ca^{2+} to zero before lysis, then determine F_{min} before F_{max}.

Since F_{max} and F_{min} at 339 nm excitation are not affected by Mg^{2+}, pH above 6.8, K^+, or Na^+, it is not necessary that these variables are the

[39] D. A. Williams and F. S. Fay, *Am. J. Physiol.* **250,** C779 (1986).

[40] J. R. Blinks, W. G. Wier, P. Hess, and F. G. Prendergast, *Prog. Biophys. Mol. Biol.* **40,** 1 (1982).

same in the lysate as inside the cells. Thus to calculate $[Ca^{2+}]_i$, only F_{max}, F_{min}, and the K_D inside the cells need to be known. One detail that is sometimes neglected is that, to obtain a true F_{min} in Ca-EGTA mixtures, it is necessary to raise the pH of the medium above 8. This is required to lower the effective K_D of EGTA for Ca^{2+} to nanomolar levels.

Calibration with Ionophore and Mn^{2+}

A method for driving the quin2 to two extreme states without actually lysing the cells is to use ionomycin, a Ca^{2+} ionophore. This calibration[41] is particularly advantageous in cells attached to coverslips and in single cells viewed by fluorescence microscopy.[42] Another useful application of this calibration is when very low quin2 loadings are used, so that the autofluorescence artifacts due to cell lysis become large compared to the quin2 signal.

The actual procedure is the following: At the end of the experiment, in the presence of extracellular Ca^{2+}, a large dose of a Ca^{2+} ionophore is added to increase $[Ca^{2+}]_i$ to several micromolar. Normally ionomycin is used instead of A23187, because the latter is fluorescent at the quin2 wavelengths. Ionomycin is now commercially available from Calbiochem (Behring Diagnostics). The fluorescence level obtained with ionomycin is assumed to be F_{max}. Mn^{2+} at ~1 m*M* is then added to quench intracellular and extracellular quin2 fluorescence. The intracellular signal can be quenched because the ionophore transports Mn^{2+} into the cells. When a new steady level is reached, usually requiring a few minutes, the residual signal should correspond to cell autofluorescence. F_{min} is then calculated according to the equation $F_{min} = 0.16(F_{max} - \text{autofluorescence})$. Note that F_{min} does not equal the autofluorescence after Mn^{2+}. The factor 0.16 (see Refs. 41 and 42) derives from the observation that, in pure dye solutions *in vitro*, F_{min} is typically 16% of F_{max}. In different samples, F_{min}/F_{max} has varied from 0.12 to 0.20. Then Eq. (1) is applied. In order to verify that the dose of Ca^{2+} ionophore is sufficient to increase $[Ca^{2+}]_i$ to F_{max}, this calibration has to be checked with the lysis method. In some cells, for example, in the insulinoma cell line RINm5F, ionophore treatment does not approach F_{max} as measured by lysis unless the ionomycin concentration is high enough to cause lysis by itself. We have occasionally observed a paradoxical opposite, that the signal with the ionophore is higher than F_{max} obtained by lysis. There are two possible explanations

[41] T. R. Hesketh, G. A. Smith, J. P. Moore, M. V. Taylor, and J. C. Metcalfe, *J. Biol. Chem.* **258,** 4876 (1983).

[42] J. Rogers, T. R. Hesketh, G. A. Smith, M. A. Beaven, J. C. Metcalfe, P. Johnson, and P. B. Garland, *FEBS Lett.* **161,** 21 (1983).

for this result: quenching of extracellular quin2 by traces of heavy metals present in the medium (see the section Correction for Extracellular Dye, Autofluorescence, and Heavy Metals, below) or incomplete hydrolysis. In this latter case it is probable that the intensity of fluorescence of unhydrolyzed or partially hydrolyzed quin2 is higher inside the cells compared to the medium.

Calibration by the Ratio of Two Excitation Wavelengths

Excitation spectra of quin2 at varying Ca^{2+} levels (Fig. 2) show maximal sensitivity to Ca^{2+} at 335–340 nm and a crossover point somewhere between 355–360 nm. At the crossover point, the dye fluorescence is independent of Ca^{2+}. The ratio of intensity at 335–340 nm excitation to that at or above the crossover wavelength reflects the Ca^{2+} level but is independent of dye concentration and path length. In principle, such ratios can be calibrated to read Ca^{2+} without requiring any lysis of the cells or use of Ca^{2+} ionophore. Ratio calibration is particularly desirable when observing single cells by microscopy. In practice, however, quin2 is poorly suited to ratio calibration, despite attempts[11] to use it in that way. The wavelength and relative amplitude of the crossover point, but not the Ca^{2+} peak are quite sensitive to free Mg^{2+} (Fig. 4) and environmental viscosity, so that these parameters must be known and matched in the calibration medium. Quin2 fades rapidly at the high illumination intensities of fluorescence microscopy, but the photoproduct(s) retains some fluorescence whose excitation ratio is different from quin2 (R. Y. Tsien, unpublished observations). Autofluorescences at the two wavelengths must be accurately deducted before forming the ratio. Such subtraction of autofluorescence is particularly difficult at the longer excitation wavelength, at which quin2 is dim but cellular fluorophores such as NADH are peaking. Boosting the excitation intensity at the longer wavelength, for example, by using a mercury instead of xenon arc, can make the short- and long-wavelength quin 2 signals more nearly equal but cannot overcome the contamination by autofluorescence. Ratio calibration only becomes practical with dyes of much greater brightness and reduced Mg^{2+} sensitivity, such as fura-2 and indo-1 (see Refs. 10 and 18–20).

Correction for Extracellular Dye, Autofluorescence, and Heavy Metals

Whichever calibration procedure one decides to use, often corrections for (1) autofluorescence, (2) extracellular dye, and (3) intracellular heavy metals need to be performed before $[Ca^{2+}]_i$ is calculated.

Autofluorescence. Correction for autofluorescence or scattered stray light due to the cells themselves is critical if one uses the ratio mode. With

the two other calibration procedures, any constant background level of autofluorescence is cancelled out in $F - F_{min}$ or $F_{max} - F$. Only changes of autofluorescence need to be deducted. Such changes are simply assessed by subjecting an aliquot of unloaded cells, at the same cytocrit as used for the loaded cells, to the entire sequence of manipulations in the cuvette. Usually the largest change in autofluorescence is observed upon cell lysis. Its effect on the calibration is greatest with low loadings of intracellular quin2.

There are a number of compounds which fluoresce at the wavelength of quin2, such as the Ca^{2+} ionophor A23187, anticalmodulin drugs such as trifluperazine, etc. Their fluorescence has to be subtracted before calibrating the signal. Other compounds are not fluorescent but quench the signal of quin2. Examples include the mitochondrial uncouplers FCCP, CCCP, and 2,4-dinitrophenol, and the microtubule inhibitor colchicine. These UV-absorbing drugs probably just attenuate the excitation beam before it reaches the zone from which the emission is collected. Excessive turbidity due to too dense a cell suspension can have a similar effect. Such inner-filter effects can be assessed by control experiments in which drugs or unloaded cells are added to quin2 in buffer. Inner filtering can be reduced by either reducing the optical density, offsetting the cuvette, decreasing its path length, or collecting emission from its front face rather than at right angles to the excitation.

Extracellular Dye. Ideally, the quin2 fluorescence signal measured should originate only from dye trapped in the cell cytoplasm. In practice, it is common to find some extracellular dye even after repeated washings. Its contribution to the overall fluorescence signal needs thus to be subtracted before calculating $[Ca^{2+}]_i$. Extracellular quin2 can derive from (a) carryover from the loading incubation, (b) cell breakage during centrifugation and resuspension of the pellet, or (c) leakage during the experiment. In this latter case, quin2 can either leak from intact cells or be released as a consequence of cell death.

Carryover (a) is a trivial problem and can be easily solved by more thorough washing. Several maneuvers can be tried to reduce extracellular quin2 to effects of (b) and (c), but it has proved impossible in our hands to eliminate it completely in cell suspensions. Extracellular quin2 due to (b) varies considerably between cell types. It is quite large (20–40%) in cell types which require trypsinization during preparation, such as in hepatocytes and pancreatic acini and in the two ascites carcinoma lines Ehrlich and Yoshida. On the other hand, extracellular quin2 is of minor relevance in lymphocytes and practically negligible in platelets and neutrophils. Compared to extracellular quin2 found immediately after spinning and resuspension, quin2 leakage during the experiment has usually proved to

be less important. We calculated a leakage rate of 10–15% of total content in 1 hr at 37° in mouse thymocytes and up to 30% under the same conditions for Ehrlich ascites carcinomas. The leakage rate is very temperature sensitive, being negligible at room temperature. Thus, after the loading we usually keep the stock suspension of cells at room temperature or even in a water bath at 10–20°.

The most obvious way to determine the extent of extracellular quin2 is to centrifuge an aliquot of the cell suspension and measure the amount of quin2 in the supernatant. This procedure is simple but tedious to repeat on every sample. Alternatively, the amount of extracellular quin2 can be determined *in situ* either by quenching with Mn^{2+} or by chelation of extracellular Ca^{2+} with EGTA. Because the Mn^{2+}–quin2 complex has negligible fluorescence, and quin2 binds Mn^{2+} about 500 times more avidly than it binds Ca^{2+},[41] Mn^{2+} concentrations $> 10^{-5}$ *M* nearly completely quench the fluorescence signal of quin2 even in the presence of millimolar concentrations of Ca^{2+}. Because Mn^{2+} has a very low permeability through the plasma membrane of resting cells in the absence of ionophore, 50–100 μM external Mn^{2+} will quench external quin2 immediately, while intracellular dye is only slowly affected. To continue the experiment, Mn^{2+} can then be chelated with a slight excess of diethylenetriaminepentaacetic acid (DTPA), a chelator whose $Mn^{2+}:Ca^{2+}$ preference, 63,000,[43] greatly exceeds that of quin2 or EGTA. This should restore fluorescence to the initial value. Sometimes the fluorescence restoration due to DTPA is larger than the quenching due to Mn^{2+}, because traces of heavy metals were already present in the medium. The opposite discrepancy, only partial restoration of fluorescence by DTPA, can occur when the membrane permeability to Mn^{2+} has been increased so that Mn^{2+} could relatively rapidly quench the intracellular signal as well. However, DTPA itself, with five negative and two positive charges, is very unlikely to enter any cell still capable of trapping quin2. Therefore the fluorescence restoration due to DTPA is still a good index of extracellular quin2. The quenching of intracellular and extracellular signal by Mn^{2+}, and their different sensitivities to DTPA can be turned into a powerful experimental tool when it is necessary to distinguish whether a rise in quin2 fluorescence is due to a rise in $[Ca^{2+}]_i$ or due to quin2 release into the medium,[41] and when studying influx through "Ca^{2+} channels" that also let Mn^{2+} or Ni^{2+} through.[44] In the first case, including Mn^{2+} in the medium before adding the stimulus will give a decrease of fluorescence if

[43] A. E. Martell and R. M. Smith, "Critical Stability Constants," Vol. 1. Plenum, New York, 1974.

[44] T. Hallam and T. J. Rink, *FEBS Lett.* **186,** 175 (1985).

quin2 leakage occurs, which will be completely reversed on DTPA addition. In the second case, the entrance of Mn^{2+} into the cells will cause a quenching of the intracellular quin2 fluorescence that cannot be reversed by DTPA.

Another way of determining extracellular quin2 is to chelate all extracellular Ca^{2+} with EGTA and a base such as Tris to raise the pH of the medium to >8. Extracellular quin2, or quin2 in leaky cells, responds immediately to a drop in extracellular Ca^{2+}, whereas intracellular quin2 should respond only gradually. Thus the immediate drop of fluorescence upon addition of EGTA + Tris is taken as a measure of extracellular quin2. Because of the change in pH required by this method, it is usually performed at the end of the experiment, just before lysis. Obviously, extracellular dye must not have been quenched by traces of heavy metals, so it is advisable to include 10^{-5}–10^{-4} *M* DTPA in the startup medium.

Figure 5 gives an example of how $[Ca^{2+}]_i$ can be calibrated in the

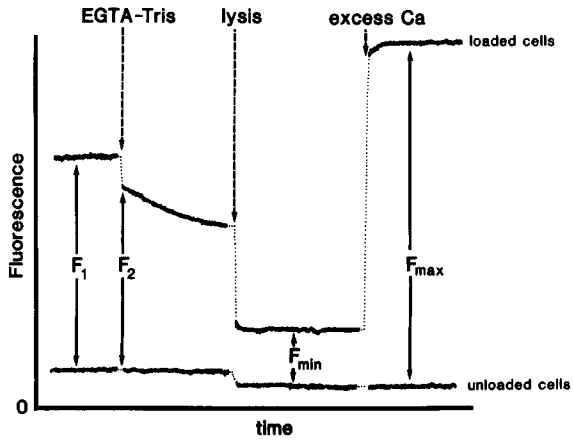

FIG. 5. Calibration of $[Ca^{2+}]_i$ with correction for leaked dye using the EGTA-Tris method. These traces are fictitious but representative of typical real records. The top trace indicates that fluorescence of quin2-loaded cells subjected to sudden chelation of extracellular Ca^{2+}, lysis, then readdition of excess Ca^{2+}. The bottom trace represents the autofluorescence of a matching aliquot of unloaded cells undergoing the same manipulatons, though in practice it would not be necessary to make the timings between additions exactly parallel to the run with loaded cells. In this example, the autofluorescence is shown as decreasing upon lysis; this actually varies with cell type. F_1, F_2, F_{min}, and F_{max} each are fluorescence amplitudes measured above autofluorescence levels. F_1 can be measured at any time desired as long as no increase in leakage happens between F_1 and F_2; F_2 should be measured as soon as possible after mixing, with back extrapolation if necessary; F_{min} and F_{max} are measured whenever their amplitudes have stabilized. Then $[Ca^{2+}]_i$ corresponding to F_1 is $K_D(F_2 - F_{min})/F_{max} - F_1)$ where K_D is the quin2 effective dissociation constant for Ca^{2+}, typically 115 n*M* under conditions representative of mammalian cytoplasm.

presence of extracellular dye, using the EGTA–Tris protocol to measure leakage. The top trace starts with intact quin2-loaded cells in medium with $>10\ \mu M$ Ca^{2+}. The lower trace represents the signal from a matching sample of unloaded cells, which will be treated with the same sequence of manipulations so that the autofluorescence contribution can be deducted at each stage. Let x be the fraction of leaked dye, and $(1 - x)$ the fraction still in the cells. The composite fluorescence F_1 due to the dye (autofluorescence having been subtracted) is $xF_{max} + (1 - x)([Ca^{2+}]_i F_{max} + K_D F_{min})/([Ca^{2+}]_i + K_D)$, where F_{max} and F_{min} as usual denote the fluorescence that the dye would have if all of it were exposed to $>5\ \mu M$ or <2 nM Ca^{2+}, respectively. Then external $[Ca^{2+}]$ is abruptly lowered to <2 nM by addition of EGTA and Tris base, then the dye fluorescence is immediately remeasured as F_2. The section How to Calculate and Prepare Ca-EGTA Buffers (see below) discusses how to compute the necessary pH rise and EGTA addition; it may be valuable to rehearse with blank medium to determine in advance how much base is needed for the pH change. If one can assume that x and $[Ca^{2+}]_i$ have not yet changed, then $F_2 = xF_{min} + (1 - x)([Ca^{2+}]_i F_{max} + K_D F_{min})/([Ca^{2+}]_i + K_D)$. The fluorescence will probably then drop gradually as x increases or $[Ca^{2+}]_i$ decreases by pumping. Upon complete lysis of the cells by any means, all the dye is exposed to very low Ca^{2+}, yielding a direct measure of F_{min}. F_{max} then results from readdition of enough Ca^{2+} to the lysate to give a reliable excess (say 1 mM) of total Ca^{2+} over EGTA. Solution of the above expressions for F_1 and F_2 gives the simple equations:

$$x = (F_1 - F_2)/(F_{max} - F_{min})$$
$$[Ca^{2+}]_i = K_D(F_2 - F_{min})/(F_{max} - F_1)$$

The latter equation is remarkably like Eq. (1) except that F_2 and F_1 replace F in numerator and denominator, respectively.

Intracellular Quenching by Heavy Metals. Some cell types, notably dedifferentiated tumor cell lines, already contain enough exchangeable heavy-metal ions to quench intracellular quin2.[13,40] Heavy metals such as Mn^{2+}, Zn^{2+}, Fe^{2+}, and Cu^{2+} not only directly suppress quin2 fluorescence but also competitively reduce Ca^{2+} binding.[36] Such quenching is not constant but tends to be relieved when $[Ca^{2+}]$ is raised very high to determine F_{max} in either the lysis or ionophore calibration procedures. It is also removed when F_{min} is attained by alkaline EGTA addition to lysates. Since intracellular heavy metals depress F more than they affect F_{max} or F_{min}, they lead to underestimation of $[Ca^{2+}]$. The error is greatest at low quin2 loadings, because the exchangeable heavy-metal pool is of limited size.

Testing for dye quenching by endogenous heavy metals is now very simple with a membrane-permeant, heavy-metal-specific chelator *N,N,N′,N′*-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN).[13] TPEN has much higher affinity for heavy metals but lower affinity for Ca^{2+} than quin2 has. Unlike DTPA, TPEN is almost uncharged at physiological pH and readily crosses membranes as the free base. TPEN can effectively strip heavy metals from quin2 both in homogeneous solution and when applied externally to liposomes containing trapped quin2 and heavy metals. A similar effect has also been demonstrated in intact cells both on endogenous as well as deliberately introduced heavy metals.[13] The amount of intracellular quenching by heavy metals varies significantly between cell types. Among the cells tested by us, the highest quenching was observed in two ascites carcinomas (Ehrlich and Yoshida) and the lowest in human neutrophils. We thus suggest that TPEN should always be tested in cell preparations when absolute values of $[Ca^{2+}]_i$ are of importance. TPEN is now commercially available from Molecular Probes and Calbiochem-Behring.

Direct evidence that tetracarboxylate Ca^{2+} indicators bind Zn^{2+} inside tumor cells has been obtained[45] with 5FBAPTA, an analog of quin2 which can be monitored by fluorine nuclear magnetic resonance. The NMR chemical shifts of 5FBAPTA, Ca-5FBAPTA, and several heavy metal–5FBAPTA complexes are different. Ehrlich ascites tumor cells showed a peak for Zn–5FBAPTA whose intensity was about 30% of the Ca–5FBAPTA peak.

Measurement of Dye Content

Measurement of the intracellular dye loading is particularly important when considering Ca^{2+} buffering or other potential perturbing effects of the dye or the loading process. Some workers present such data only in terms of initial ester concentration in the suspension, but the final cellular content of de-esterfied dye is obviously more directly relevant. The latter needs actually to be measured because it depends not only on the nominal ester concentration but on the purity of the ester, the exact loading protocol, and any subsequent dye leakage.

Intracellular dye content is most simply determined by measuring $(F_{max} - F_{min})$ from the cells and comparing it to the $(F_{max} - F_{min})$ obtained from one or more samples of quin2 free acid of known concentration, analogously exposed to saturating versus zero Ca^{2+}. The comparison establishes the concentration of quin2 in the cell lysate or suspension. One

[45] J. C. Metcalfe, T. R. Hesketh, and G. A. Smith, *Cell Calcium* **6**, 183 (1985).

then needs to know the cytocrit, or ratio of cell volume to total suspension volume. Typically, this is estimated by hemacytometer or Coulter counting of the cells before lysis, together with some estimate for the average volume per cell. The prelysis concentration of dye in the cells is then just the lysate or suspension concentration divided by the cytocrit. Thus if lysate ($F_{max} - F_{min}$) corresponds to 1 μM dye, and the cells had occupied 0.1% of the suspension volume, the dye content was 1 mM. In this example and all of our work, such concentrations are with respect to total cell volume, though they could be expressed in moles per liter of cytosolic volume or intracellular water if one had the appropriate values per cell.

Some authors have preferred to estimate loadings using radioactively labeled dye and scintillation counting.[41] This approach could be valuable in tissues that are too pigmented or autofluorescent for the standard method. Otherwise, tracer labeling would seem to offer little advantage, since the radioactive material is much more costly than ordinary dye and requires additional data on radiochemical purity, specific activity, and counting efficiency.

Side Effects of Quin2 Loading

Quin2 loading often affects cell function, especially at higher levels of dye content. Therefore it is always desirable to check the relevant physiological functions of the tissue, especially acute responses that can be measured in the same time scale as the quin2 fluorescence signals. Most known effects of quin2 loading are probably due to one of three obvious mechanisms, in order of increasing prevalence: (1) heavy metal chelation by quin2; (2) toxicity or acidification induced by the formaldehyde and acetic acid by-products of acetoxymethyl (AM) ester hydrolisis; (3) Ca^{2+} buffering by quin2.

1. Just as intracellular quin2 fluorescence can be perturbed by heavy metals, the dye could be chelating heavy metal ions that are potentially important in cell function.[13] The obvious test for such a mechanism would be to see if the membrane-permeant heavy metal chelator TPEN has similar effects on the cell. Because TPEN has higher affinities than quin2 has for heavy metals, and TPEN can probably reach all compartments of the cell, TPEN efficacy should exceed that of quin2. Failure of TPEN to affect cell function probably rules out heavy metal chelation as a mechanism for quin2, whereas a finding of TPEN activity is more ambiguous. Because of this ambiguity and the only recent commercial availability of TPEN, heavy metal chelation has not yet been proven as the mechanism for any particular side effect of quin2 loadings.
2. Each molecule of quin2/AM eventually generates four molecules of

formaldehyde, four of acetate, and eight protons upon full hydrolysis.[16] The acetate ions are probably innocuous and carry away four of the protons as they diffuse out of the cell. The remaining protons ought still to be easily managed by the normal pH-regulatory mechanism of the cell, though instances have been reported[46] in which a small depression of internal pH persists. Such acidification may really be an indirect consequence of the formaldehyde, which is undoubtedly the most worrisome side product. Fortunately, the formaldehyde is generated gradually and may partly be able to diffuse out of the cell. The most clearly analyzed consequence of the formaldehyde produced by AM ester hydrolysis is inhibition of glycolysis in human red cells due to formaldehyde depletion of NAD and formation of NADH.[47] A recommended remedy[48] for this effect is to include several millimolar pyruvate in the medium. Pyruvate can not only reoxidize NADH but also serve as an alternative substrate for oxidative phosphorylation in cells with mitochondria. A further defense against other reactions of formaldehyde is ascorbate at several hundred micromolar. Ascorbate is known to block for example, the reactions of formaldehyde with lysine residues.[49] Pyruvate and ascorbate together seem sufficient to protect even retina, a tissue notoriously sensitive to formaldehyde (G. Ratto, W. G. Owen, and R. Y. Tsien, unpublished results; see also Ref. 15). Further agents that might be beneficial include thiols such as mercaptoethanol, dithiothreitol, or permeant glutathione esters, though such agents alone were ineffective in erythrocytes.[47]

An additional test for toxicity due to AM ester by-products is to use an AM ester of a carboxylic acid with negligible Ca^{2+} affinity. A particularly realistic model is the acetoxymethyl ester of *o*-anisidine-*N*,*N*′-diacetic acid ("anis1/AM"[5] or "APDA AM ester"[41]). Because anis1/AM represents one isolated half of the quin2/AM molecule, it should be given at twice the concentration of quin2/AM. Indeed, anis1/AM can reproduce the lowering of cellular ATP sometimes caused by quin2/aM. Unfortunately, anis1/AM is not yet commercially available. Less realistic but more accessible agents for comparison are methylene diacetate and extracellular formaldehyde itself.

An ideal solution to the formaldehyde problem would be to replace the acetoxymethyl ester group by another α-acyloxyalkyl group. For example, α-acetoxyethyl esters would release acetaldehyde, whose lesser toxicity than formaldehyde is reflected in our ability to imbibe ethanol not

[46] D. C. Spray, J. Nerbonne, A. Campos de Carvalho, A. L. Harris, and M. V. L. Bennett, *J. Cell Biol.* **99,** 174 (1984).
[47] T. Tiffert, J. Garcia-Sancho, and V. L. Lew, *Biochim. Biophys. Acta* **773,** 143 (1984).
[48] J. Garcia-Sancho, *J. Physiol. (London)* **357,** 60P (1984).
[49] L. Trezl, I. Rusznak, E. Tyihak, T. Szarvas, and B. Szende, *Biochem. J.* **214,** 289 (1983).

methanol. α-Acetoxyethyl esters of simple carboxylic acids and even of anisl do hydrolyze satisfactorily, but when applied to all four carboxylates of quin2, the extra carbon atoms reduce solubility to an unacceptable degree and prevent significant loading.

3. Ca^{2+} buffering, the most common and most inherent side effect of quin2 loading, has already been discussed in the Introduction. Obscure side effects can result from Ca^{2+} buffering, for example, inhibition of Na_i/Ca_o exchange in squid axons.[50] This effect is equally well produced by EGTA and appears to be a consequence of slowing a rise in $[Ca^{2+}]_i$. The normal mode of operation of the exchanger in resting cells, Na_o/Ca_i exchange, is not affected by quin2 or EGTA.

The Ca^{2+} buffering caused by quin2 can be highly useful if properly controlled and varied. Valuable information can be deduced concerning the origin and the role of the $[Ca^{2+}]_i$ rises induced by agonists. For example, if a $[Ca^{2+}]_i$ rise originates only from an intracellular pool of limited capacity, increasing intracellular quin2 should change the amplitude of the rise but hardly affect its kinetics.[4,6] Furthermore, by measuring the amplitude of the $[Ca^{2+}]_i$ rises at different intracellular quin2 concentrations, it should also be possible to calculate the actual amount of Ca^{2+} released and the endogenous Ca^{2+} buffering capacity of the cell.[5] However, if the $[Ca^{2+}]_i$ rise is due to the establishment of a new steady-state level of Ca^{2+} influx and efflux at the plasma membrane, then quin2 should increase the time required to attain the steady state, but not affect the level eventually achieved. Most agonists often act both on the influx and on the release of Ca^{2+} from stores, and the effect of quin2 loading is a mixture of the two. Another effect sometimes observed with high concentrations of quin2 is that the release from stores and the influx from the extracellular medium become well separated kinetically, since the release from stores is completed in less than 10 sec while the influx continues for several minutes. Varying the amount of quin2 in the cell is not only useful to study the mechanism of the $[Ca^{2+}]_i$ rises induced by agonists, but also to study the relationships between Ca^{2+} and cell activation. In fact, if other side effects can be excluded, then the effect of Ca^{2+} buffering on cell responses shows whether Ca^{2+} was important in that response. A further effective manipulation to study the causal relationship between $[Ca^{2+}]_i$ and cellular responses is to load the cell with quin2 in the absence of Ca^{2+} in the extracellular medium. Under these conditions, the cells have no Ca^{2+} with which to titrate the dye to the normal resting level, so quin2 loading will result in a dramatic reduction of the resting $[Ca^{2+}]_i$.[4,6-8] Surprisingly, this use of quin2, i.e, as a Ca^{2+} buffer as well as a Ca^{2+} indica-

[50] T. J. A. Allen and P. F. Baker, *Nature (London)* **315**, 755 (1985).

tor, has found little attention among investigators other than the present authors and their collaborators. Although newer Ca^{2+} indicators such as fura-2 and indo-1 may supersede quin2 as a Ca^{2+} indicator, quin2 may remain important as an intracellular Ca^{2+} buffer because it is easier than the new indicators to load into cells at high concentrations. In conclusion, quin2 loading can inherently alter the kinetics and/or the amplitude of $[Ca^{2+}]_i$ changes, but this perturbation can be turned into an invaluable tool if used appropriately.

How to Calculate and Prepare Ca-EGTA Buffers

Practically all methods for measuring or controlling intracellular free Ca^{2+} require reference media with $[Ca^{2+}]$ well buffered at low levels. Usually, such buffers are based on the Ca^{2+} chelator EGTA, ethylene glycol bis(2-aminoethyl)ether *N,N,N′,N′*-tetraacetic acid. Other buffers are known[12] with the same Ca : Mg selectivity as EGTA but lesser pH sensitivity, faster kinetics, UV indicator properties, and a range of Ca^{2+} affinities, but they are not used for routine reference or extracellular solutions because they are very much more expensive than EGTA and are not as indefinitely stable at room temperature. Despite the central importance of accurate EGTA buffers in work on cellular Ca^{2+}, beginning workers often have difficulty finding a coherent description of how to make them. Many are under the misapprehension that elaborate computer programs[51,52] are necessary to prepare buffer recipes. In fact, such programs are rarely necessary. Far more important is a basic understanding of the chelation chemistry of EGTA and the possible sources of systematic error in solution preparation.

EGTA is widely available from most chemical suppliers and is an amorphous white powder practically insoluble in water. We have used the "puriss." grade of Fluka, with which we have had no difficulties. (For detailed comparison of EGTAs from different manufacturers, see Refs. 53 and 54.) None of the commercial products are >99.5% pure, but traditionally no one purifies EGTA further. Convenient stock solutions of EGTA are 0.1–1.0 *M* and are simply prepared by adding sufficient base [NaOH, KOH, $Me_4N^+OH^-$, tris(hydroxymethyl)aminomethane, or whatever base provides the desired cation] to dissolve all the EGTA and reach a pH of 7–8, then diluting to the appropriate final volume. With a strong base such as KOH, approximately two equivalents are required, resulting in a solu-

[51] D. D. Perrin and I. G. Sayce, *Talanta* **14,** 833 (1967).
[52] A. Fabiato and F. Fabiato, *J. Physiol. (Paris)* **75,** 463 (1979).
[53] D. M. Bers, *Am. J. Physiol.* **242,** C404 (1982).
[54] D. J. Miller and G. L. Smith, *Am. J. Physiol.* **246,** C160 (1984).

tion of K_2H_2EGTA since EGTA starts out with four titratable protons. The dissolution should proceed smoothly as base is stirred in; if granules appear that dissolve only very slowly even after the supernatant pH is >7, impurities are suspected.

Obviously the above Ca-free stocks of EGTA are convenient for diluting into the other constituents of physiological media. To prepare a given Ca-EGTA mixture with defined free $[Ca^{2+}]$, the simplest approach is merely to add the correct amount of $CaCl_2$, whose amount is calculated as described below. This procedure has practical drawbacks. Mixing $CaCl_2$ with K_2H_2EGTA gives $K_2CaEGTA + 2H^+ + 2Cl^-$. The large acid production and pH sensitivity of EGTA require that solutions be retitrated to the correct pH after mixing. An even more serious problem concerns the accuracy of the $[Ca^{2+}]$ setting when the amount of Ca begins to approach that of the EGTA. Small errors in the precise titer of either the Ca or EGTA stock can cause enormous swings in final free $[Ca^{2+}]$. So, if one wishes to prepare a range of Ca-EGTA buffers, it is usually worthwhile adopting a different approach, the preparation of a concentrated stock solution of $K_2CaEGTA$ in which the Ca and EGTA contents are verified to be within 0.5% of each other. This approach is much more accurate than the first for preparing final solutions where Ca is a large fraction of EGTA. It relies on the availability of sensitive methods for assessing whether total Ca is in excess or deficit compared to EGTA in a concentrated solution. One obvious method is to use a precalibrated Ca^{2+}-selective electrode; concentrated EGTA is titrated with $CaCl_2$ until an inflection point is seen in the plot of $\log[Ca^{2+}]$ versus $CaCl_2$ added. At this inflection point, $[Ca^{2+}]$ should be close to the geometric mean of the total EGTA concentration and the effective K_D of EGTA for Ca^{2+} at the existing pH.

A less obvious but often more convenient method for titrating Ca against EGTA was first described by Moisescu and Pusch.[55] Their "pH-metric" method requires only a pH electrode, not a Ca^{2+}-selective electrode. It relies on the fact that if EGTA at pH 6–9 is in excess over Ca, an addition of Ca will then acidify the solution. Once Ca is in excess over EGTA, adding further Ca negligibly affects the pH. The following is a typical recipe for 10 ml of 1.0 *M* stock $K_2CaEGTA$, in which excess KCl is minimized: 3.84 g EGTA free acid (1% over the theoretical weight of 10 mmol, to allow for the typical purity of 99%), about 0.95 g $CaCO_3$ (analytical grade, 9.5 mmol), and 19 mmol solid KOH (about 1.20 to 1.25 g of 85% pellets) and 6 ml H_2O are mixed in a beaker, stirred, heated to 90–100° until CO_2 evolution ceases, then returned to room temperature. Concen-

[55] D. G. Moisescu and H. Pusch, *Pfluegers Arch.* **355,** R122 (1975).

trated aqueous KOH (the commercially available 45% solution is convenient) is cautiously added in 10-μl portions until the pH reaches 7 to 8. Almost all the solids should have dissolved. Then the solution pH is noted before and after addition of $CaCl_2$ in 0.01- to 0.05-mmol aliquots, for example, 10–50 μl of a 1 *M* solution. Assuming the pH has dropped, KOH is then added to restore the pH; about 2 mmol per mmol Ca should be required. Precise adjustment of pH is not essential so long as it remains between 6.5 and 8. The process of Ca addition and pH restoration is repeated. It is worth keeping a log of how many Ca additions have been made and what pH change each produced. Eventually, the pH decrement per unit of Ca added decreases fairly abruptly. We stop adding Ca when $\Delta pH/\Delta Ca$ falls below one-half of its original value. The mixture is transferred to a 10-ml volumetric flask. Several small portions of water are used to rinse the pH electrode and beaker and are added to the volumetric flask to complete the transfer and reach the 10-ml mark. After mixing the flask contents, residual haziness is removed by membrane filtration.

For most reasonable buffer compositions, a few simple steps on a hand calculator are sufficient to calculate how much Ca^{2+}, Mg^{2+}, and EGTA should be mixed to achieve a desired free $[Ca^{2+}]$ and $[Mg^{2+}]$. A simple understandable procedure is preferable to a black-box computer program whose operations cannot be checked. Our first step is to obtain the effective dissociation constants of EGTA for Ca^{2+} and Mg^{2+} at the desired pH and temperature. This discussion refers to dissociation constants instead of association constants merely because units of real concentration are more easily remembered and interpreted than values in liters/mole, which are prone to errors in carrying exponents. The values used in our laboratory are listed in Table I at 0.05 − 0.1 pH unit intervals from 6.50 to 8.20 at 20° and 37° in 0.1 *M* ionic strength, and at 18° in 0.25 *M* ionic strength.

Once the effective dissociation constants $K_D(Ca)$ and $K_D(Mg)$ at the relevant pH are known, Ca^{2+}-EGTA buffers are governed by the following equations, where $[EGTA_f]$ is defined as [all forms of EGTA not bound to Ca^{2+} or Mg^{2+}] = $[EGTA^{4-}] + [H \cdot EGTA^{3-}] + [H_2EGTA^{2-}]$. Likewise [MgEGTA] is defined as [all MgEGTA complexes] = $[MgEGTA^{2-}] + [MgHEGTA^{-}]$.

$$[CaEGTA] = [Ca^{2+}][EGTA_f]/K_D(Ca) \quad (2)$$

$$[MgEGTA] = [Mg^{2+}]]EGTA_f]/K_D(Mg) \quad (3)$$

$$\text{Total EGTA} = [EGTA_f] + [CaEGTA^{2-}] + [MgEGTA] \quad (4)$$

$$\text{Total Ca} = [Ca^{2+}] + [CaEGTA^{2-}] \approx [CaEGTA^{2-}] \quad (5)$$

$$\text{Total Mg} = [Mg^{2+}] + [MgEGTA] \quad (6)$$

The key to easy calculation is to decide first on $[EGTA_f]$, then work out

what total EGTA, Ca, and Mg are required. This contrasts with the traditional procedure of fixing total EGTA then calculating total Ca and Mg, a masochistic exercise in simultaneous nonlinear equations that is largely responsible for the fear and trembling with which these equilibria are often viewed.

Suppose we desire a buffer with 100 n*M* free Ca^{2+} and 1 m*M* free Mg^{2+} at 37°, pH 7.05. From Table I, K_D(Ca) is 213 n*M* and K_D(Mg) is 9.65 m*M*. Suppose we choose [$EGTA_f$] = 2 m*M*. Then [CaEGTA] = 100 n*M* × 2 m*M*/213 n*M* = 0.939 m*M*. Also, [MgEGTA] = 1 m*M* × 2 m*M*/9.65 m*M* = 0.207 m*M*. Therefore the total EGTA required is 2 m*M* + 0.939 m*M* + 0.207 m*M* = 3.146 m*M*. The total Ca required is 0.939 m*M* + 100 n*M* = 0.9391 m*M*. The total Mg required is 0.207 m*M* + 1 m*M* = 1.207 m*M*.

Suppose we now wish to raise free Ca^{2+} in the preceding solution to 1 μM while maintaining pH and free Mg^{2+} constant. Obviously we need to increase [CaEGTA] by 900 n*M* × 2 m*M*/213 n*M* = 8.45 m*M*. This is best added from a concentrated stock solution of K_2CaEGTA whose Ca and EGTA contents are accurately balanced and whose pH is already near 7–8. If instead one tried to add 8.45 m*M* $CaCl_2$ and 8.45 m*M* K_2H_2EGTA, a small percentage error in either concentration would lead to a much larger percentage error in [$EGTA_f$] and the actual [Ca^{2+}] achieved. Also, about 16.9 m*M* H^+ would be generated, requiring additional careful tritration to restore pH to 7.05.

Note that by adding Ca in conjunction with EGTA, we preserve [$EGTA_f$] and avoid disturbing the Mg^{2+} equilibria. If one added $CaCl_2$ without EGTA, [$EGTA_f$] would fall and [free Mg^{2+}] would rise, though not above 1.207 m*M*.

It is no more difficult to calculate how much EGTA to add to reduce Ca^{2+} from extracellular to intracellular levels. Suppose the starting medium has 1.00 m*M* Ca^{2+} and 0.50 m*M* Mg^{2+}. What do we add to reach 200 n*M* free Ca^{2+} and 0.8 m*M* free Mg^{2+} at pH 7.00, 20°? Once we add the EGTA, essentially all the 1 m*M* Ca^{2+} (actually, 1 m*M*–200 n*M* = 0.9998 m*M*) will become CaEGTA. From Table I, K_D(Ca) and K_D(Mg) are 376 n*M* and 33.9 m*M*, respectively. From Eq. (2), [$EGTA_f$] needs to be K_d(Ca)[CaEGTA]/[free Ca^{2+}] = 376 n*M* × 1 m*M*/200 n*M* = 1.88 m*M*. Then [MgEGTA] will be 0.8 m*M* × 1.88 m*M*/33.9 m*M* = 0.044 m*M*. Therefore total EGTA to be added is 1.0 m*M* + 1.88 m*M* + 0.044 m*M* = 2.924 m*M*, while total Mg is 0.844 m*M* or 0.344 m*M* extra. Of course, pH must be checked and adjusted *after* the above additions have been made.

This type of analysis is readily extended to buffers containing other ligands as well as EGTA, provided that the input concentration values refer to free cation, free ligand, or a specific complex, not to total cation or ligand. For example, suppose we want 500 n*M* free Ca^{2+}, 2 m*M* free

TABLE I
DISSOCIATION CONSTANTS OF EGTA FOR Ca^{2+} AND Mg^{2+}[a]

pH	K_D(Ca) (n*M*)			K_D(Mg) (m*M*)		
	20°, 0.1 *M*	37°, 0.1 *M*	18°, 0.25 *M*	20°, 0.1 *M*	37°, 0.1 *M*	18°, 0.25 *M*
6.5	3728	2646	5273	119.3	41.7	339
6.6	2354	1672	3331	93.5	32.4	267
6.70	1487	1057	2104	73.1	25.1	209
6.75	1182	841	1673	64.5	22.0	185.3
6.80	940	669	1330	56.9	19.25	163.9
6.85	747	532	1057	50.1	16.84	144.9
6.90	594	423	841	44.0	14.70	127.9
6.95	472	337	668	38.7	12.80	112.9
7.00	376	268	532	33.9	11.13	99.4
7.05	299	213	423	29.7	9.65	87.5
7.10	238	170.0	336	26.0	8.35	76.9
7.15	189.1	135.4	268	22.7	7.21	67.5
7.20	150.5	170.9	213	19.73	6.21	59.2
7.25	119.8	86.0	169.6	17.15	5.34	51.7
7.30	95.4	68.6	135.0	14.87	4.57	45.2
7.35	76.0	54.7	107.5	12.86	3.91	39.4
7.40	60.5	43.7	85.7	11.10	3.33	34.3
7.45	48.2	34.9	68.3	9.52	2.83	29.7
7.50	38.5	27.9	54.4	8.20	2.40	25.8
7.6	24.5	17.88	34.7	6.00	1.709	19.2
7.7	15.61	11.49	22.1	4.34	1.207	14.2
7.8	9.99	7.42	14.1	3.11	0.846	10.35
7.9	6.41	4.82	9.08	2.20	0.590	7.49
8.0	4.13	3.15	5.85	1.547	0.409	5.37
8.1	2.68	2.08	3.79	1.080	0.284	3.82
8.2	1.75	1.39	2.47	0.751	0.197	2.70

[a] $K_D(Ca) = [1 + \text{antilog}(9.58 - pH) + \text{antilog}(18.54 - 2pH)]$ ($10^{-10.97}$ *M*) at 20°, ionic strength (*I*) = 0.1 *M*. $K_D(Ca) = [1 + \text{antilog}(9.34 - pH) + \text{antilog}(18.06 - 2pH)]$ ($10^{-10.64}$ *M*) at 37°, *I* = 0.1 *M*. $K_D(Ca) = [1 + \text{antilog}(9.537 - pH) + \text{antilog}(18.498 - 2pH)]$ ($10^{-10.777}$ *M*) at 18°, *I* = 0.25 *M* (0.2 *M* KCl + 0.05 *M* NaCl) (see Ref. 10). $K_D(Mg) = [1 + \text{antilog}(9.58 - pH) + \text{antilog}(18.54 - 2pH)]$ ($10^{-5.21}$ *M*)/$[1 + \text{antilog}(7.73 - pH)]$ at 20°, *I* = 0.1 *M*. $K_D(Mg) = [1 + \text{antilog}(9.34 - pH) + \text{antilog}(18.06 - 2pH)]$ ($10^{-5.41}$ *M*)/$[1 + \text{antilog}(7.49 - pH)]$ at 37°, *I* = 0.1 *M*. $K_D(Mg) = [1 + \text{antilog}(9.537 - pH) + \text{antilog}(18.498 - 2pH)]$ ($10^{-4.567}$ *M*)/$[1 + \text{antilog}(7.885 - pH)]$ at 18°, *I* = 0.25 *M* (0.2 *M* KCl + 0.05 *M* NaCl) (see Ref. 10). The parameters for 0.1 *M* ionic strength are derived from the fundamental binding constants listed in the critical compilation of Martell and Smith.[43] The effective dissociation constant of EGTA for Ca^{2+}, K_D(Ca), is defined as [free Ca^{2+}][all forms of EGTA not bound to Ca^{2+} or Mg^{2+}]/[CaEGTA complex]. At pH >4, there are three relevant forms of metal-free EGTA: the tetraanion $EGTA^{4-}$, the singly protonated species $(H \cdot EGTA)^{3-}$, and the doubly protonated species

Mg^{2+}, 4 mM MgATP, 2 mM [$EGTA_f$] at pH 7.10, 20°. Table I gives K_D(Ca) = 238 nM, K_D(Mg) = 26.0 mM, and the analogous effective values for ATP binding to Ca^{2+} and Mg^{2+} are 226 and 116 μM, respectively, from the absolute values in the Martell and Smith compendium.[43] [CaEGTA] = 500 nM × 2 mM/238 nM = 4.20 mM; [MgEGTA] = 2 mM × 2 mM/26.0 mM = 0.154 mM; [ATP_f] = [MgATP] × 116 μM/[Mg^{2+}] = 4 mM × 116 μM/2 mM = 232 μM; [CaATP] = 500 nM × 232 μM/226 μM = 513 nM. Total EGTA = 2 mM + 4.20 mM + 0.154 mM = 6.354 mM; total ATP = 0.232 mM + 4 mM + 513 nM = 4.2325 mM; total Ca = 4.20 mM; total Mg = 2 mM + 0.154 mM + 0.232 mM = 2.386 mM.

Complex programs are needed only if total cation or ligand is prespecified. The main occasion when this is necessary is the evaluation of the effect of a given error in the assumed binding constant or in the composition of a buffer. Thus if total Ca, total Mg, and total EGTA are given as Ca_T, Mg_T, and L_T, free Ca^{2+} and Mg^{2+} are obtained from the coupled quadratic equations:

($H_2EGTA)^{2-}$. These protonations are described by conventional Henderson–Hasselbach equations, so that [H · $EGTA^{3-}$] = [$EGTA^{4-}$]$10^{(pK_1 - pH)}$ and [H_2EGTA^{2-}] = [H · $EGTA^{3-}$]$10^{(pK_2 - pH)}$ = [$EGTA^{4-}$]$10^{(pK_2 + pK_1 - 2pH)}$, where pK_1 and pK_2 are the pK_a values for the first and second protons, respectively. Therefore, K_D(Ca) = [Ca^{2+}][$EGTA^{4-}$ + $HEGTA^{3-}$ + H_2EGTA^{2-}]/[$CaEGTA^{2-}$] = [1 + $10^{(pK_1 - pH)}$ + $10^{(pK_2 + pK_1 - 2pH)}$] [$EGTA^{4-}$][Ca^{2+}]/[$CaEGTA^{2-}$] = [1 + $10^{(pK_1 - pH)}$ + $10^{(pK_2 + pK_1 - 2pH)}$]/K_{Ca}. In the above, K_{Ca} ≡ [$CaEGTA^{2-}$]/[Ca^{2+}][$EGTA^{4-}$] is the "absolute affinity" of EGTA for Ca^{2+}, achieved at very high pH where the protonations occur to a negligible extent. K_{Ca} is $10^{10.97}$ M^{-1} at 20°, 0.1 M ionic strength; pK_1 is 9.58 and pK_2 is 8.96. Note that the values for pK_1 and pK_2 are each 0.11 unit higher than the values actually listed in the tables for EGTA. The need for the 0.11 correction is explained on pp. XI–XII of Martell and Smith.[43] In brief, the original value of 9.47 for pK_1 meant that [H · $EGTA^{3-}$] = [$EGTA^{4-}$][H^+]$10^{9.47}$ M^{-1}. Now, pH as operationally used by biologists is not exactly −log[H^+] as stated in elementary texts. Rather, pH = −log[H^+ activity] = −log(0.78[H^+]) at 0.1 M ionic strength by National Bureau of Standards convention. Therefore [H^+] = $(10^{-pH})/0.78$ = $10^{0.11 - pH}$, so [H · $EGTA^{2-}$] = [$EGTA^{4-}$]$(10^{9.58 - pH})$. The same argument applies for pK_2 = 8.96 corrected from 8.85.

The effective dissociation constant for Mg^{2+} is slightly more complicated because the complex (MgH · $EGTA)^-$ is significant as well as $(MgEGTA)^{2-}$. From Martell and Smith[43] again, [$MgHEGTA^-$] = [Mg · $EGTA^{2-}$]$10^{(7.73 - pH)}$ at 20°, and K_{Mg} ≡ [$MgEGTA^{2-}$]/[Mg^{2+}][$EGTA^{4-}$] = $10^{5.21}$ M^{-1}. Therefore K_d(Mg) = [1 + $10^{(pK_1 - pH)}$ + $10^{(pK_2 + pK_1 - 2pH)}$]/K_{Mg}[1 + $10^{(7.73 - pH)}$]. Values at 37° were calculated by adding $(\Delta H)(2.303R)^{-1}[(293°K)^{-1} - (310°K)^{-1}]$ = (0.041 mol/kcal)(ΔH) to the log stability constants at 20°. Data for the behavior of EGTA at other ionic strengths (reviewed in Ref. 10; see also Ref. 57) are scanty, and theoretical extrapolations using Debye–Hückel theory are of dubious value for a complicated zwitterion like EGTA.

$$0 = [Ca^{2+}]^2 + (L_T - Ca_T + K_D(Ca) + K_D(Ca)[Mg^{2+}]/K_D(Mg))[Ca^{2+}] - (K_D(Ca))(Ca_T)(1 + [Mg^{2+}]/K_D(Mg))$$

$$0 = [Mg^{2+}]^2 + (L_T + Mg_T + K_D(Mg) + K_D(Mg)[Ca^{2+}]/K_D(Ca))[Mg^{2+}] - (K_D(Mg))(Mg_T)(1 + [Ca^{2+}]/K_D(Ca))$$

We are indebted to V. L. Lew for showing that these equations are efficiently solved by iterative refinement. Make a guess at $[Mg^{2+}]$, solve the first quadratic equation for $[Ca^{2+}]$, substitute that in the second equation, solve for $[Mg^{2+}]$, resubstitute that in the first equation again, etc. Repeat this cycle until successive rounds give negligible change in the values of Ca^{2+} and Mg^{2+}. Convergence is usually rapid.

Because EGTA is so pH-dependent, pH should be checked in the final solutions, not assumed to stay as initially set by pH buffers in the medium. Also, the pH electrode combination should be stable[56] and should have been calibrated with buffers thermostatted at the same temperature as the test solution, since the temperature-compensating knob on most pH meters only corrects the slope, not the potential offset of an electrode. Commercial pH buffers tend to be of indefinite age and extent of CO_2 pickup, sometimes disagree with one another, and are not always specified at other than room temperature, so we prefer to prepare fresh pH standards from the well-known Natural Bureau of Standards recipes.

[56] J. A. Illingworth, *Biochem. J.* **195,** 259 (1981).
[57] R. DiPolo, H. Rojas, J. Vergara, R. Lopez, and C. Caputo, *Biochim. Biophys. Acta* **728,** 311 (1983).

[15] Analyzing Transport Kinetics with Desk-Top Hybrid Computers

By HAROLD G. HEMPLING

Introduction

Modern technology has converted the powerful and rapid calculating resources of the modern computer into a desk-top unit, easily accessible to the bench scientist. The purpose of this chapter is to describe several programs which may be used on a routine basis to acquire kinetic data, to manipulate the data, and to optimize parameters. These programs are based on well-established kinetic equations from the literature and serve